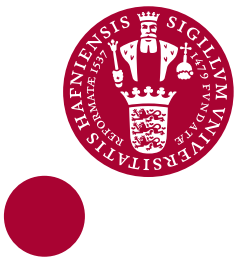

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INTRACELLULAR LOCATION OF ACETALDEHYDE METABOLISM: NON-ADH MEDIATED ETHANOL OXIDATION AS A TOOL

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Abstract

Acetaldehyde may be oxidized in rat liver both by mitochondrial and by cytoplasmic enzymes. It is of interest to know the ratio between the contribution of the two compartments, as this information is essential in the discussion of transport of reducing equivalents into the mitochondria. This problem has been investigated on isolated rat liver parenchymal cells by studies of the incorporation of ^{3}H from ^{3}H-labeled ethanol into lactate and β-hydroxybutyrate. Cells were incubated with ethanol (65 mM) and the ADH-mediated ethanol oxidation was inhibited (about 96%) by addition of pyrazole (18 mM). Preliminary results showed no incorporation of ^{3}H in lactate during the period of labeling (1 min), whereas the incorporation in β-hydroxybutyrate corresponds to half the ^{3}H-label present in the amount of ethanol oxidized to acetate. This observation indicates that acetaldehyde formed by the non-ADH mediated ethanol oxidation (about 1.4 μmol x min^{-1} x g cells wet wt.) is metabolized exclusively in the mitochondria.

Introduction

When ethanol is metabolized, acetaldehyde and NADH are produced in the cytosolic compartment of the liver cell. In addition, the oxidation of acetaldehyde to acetate is associated with the formation of NADH.

The mitochondrial membrane is relatively impermeable to NADH and the transfer of reducing equivalents from cytosol into mitochondria is dependent on indirect routes, i.e., hydrogen shuttles. The rate of production of NADH in the cytoplasmic compartment and the involvement of the shuttles in the transfer of reducing equivalents into mitochondria will

thus depend on whether the oxidation of acetaldehyde occurs in the cytosol or in the mitochondria of the liver cell. An exclusively mitochondrial oxidation of acetaldehyde will halve the cytosolic production of NADH due to the metabolism of ethanol. Processes in the cytosol may proceed to a sufficient extent to reoxidize NADH formed by the action of alcohol dehydrogenase, and the consequences of an intramitochondrial oxidation of acetaldehyde may be that no mechanism for transport of hydrogen is needed for ethanol metabolism (1).

Aldehyde dehydrogenases are located both intra- and extramitochondrially (2-4). The participation of these enzymes in acetaldehyde metabolism is, however, at present not clear. Using isolated rat liver parenchymal cells, we have tried to investigate the problem of intracellular location of the acetaldehyde oxidation.

Methods

Liver cells were prepared according to Quistorff et al. (5) from female Wistar rats, weighing 250 g, fasted for 24 hours.

Cells were incubated in Hank's solution containing 1.2 mM $CaCl_2$, 1% serum albumin, 8 mM glucose, 10 mM phosphate and 25 mM HEPES at pH 7.4. Incubations were carried out at 37°C, with atmospheric air as gas phase. The cell concentration was 3-5% (ml packed cells/ml buffer). Total incubation volume was 2.34 ml. After thermoequilibration for 5 min, [1-^{3}H]ethanol at a specific activity of 160-170 cpm/nmol was added to give a final concentration of 60 mM. After another five minutes the incubation mixture was deproteinized with perchloric acid and neutralized to pH 7.0. The samples were than centrifuged and the supernatant freeze-dried for 24 hours.

The residue was dissolved in 100 µl H_2O, 12 µl of which was chromatographed on cellulose-TL-plates in butan-2-ol and 2 N NH_3, H_2O (80:20, v/v) for 2 hours. The area between R_f = 0.21 and R_f = 0.42 which contained lactate and β-hydroxybutyrate, separated from TCA-intermediates, NADH, phosphate-esters and fatty acids, was scraped off, and eluted with 2.2 N perchloric acid and neutralized to pH 7.0. The neutralized eluate (3 ml) was divided into three equal parts. To the first part was added 200 µl $Ba(OH)_2$ and 200 µl $Al_2(SO_4)_3$. This treatment caused precipitation of lactate while β-hydroxybutyrate remained in solution. To the second part

hydrazine (0.1 M) was added (pH 9) and the mixture incubated with NAD^+, LDH and carrier lactate (1 mM) until a constant absorbance at 340 nm was attained. The mixture was precipitated in the same way as the first part of the neutralized eluate.

The difference in radioactivity between the supernatant of the first and second parts of the neutralized eluate [after precipitation with $Ba(OH)_2$ and $Al_2(SO_4)_3$] represents the radioactivity of lactate, as NADH remained in solution after $Ba(OH)_2$ and $Al_2(SO_4)_3$ precipitation.

The third part was incubated with hydrazine, NAD^+, β-hydroxybutyrate dehydrogenase and carrier β-hydroxybutyrate at pH 9 until a constant absorbance at 340 nm was attained. The mixture was taken to dryness in an evaporater, dissolved in 50 µl H_2O and rechromatographed in the system mentioned above. The area between $R_f = 0$ and $R_f = 0.21$ contained NADH with the ^3H label specifically transferred from β-hydroxybutyrate. The area was scraped off and counted, giving the radioactivity in β-hydroxybutyrate. The recovery of lactate and β-hydroxybutyrate was 90-95%. The amount of lactate and β-hydroxybutyrate in the incubation mixture was determined in parallel flasks. Ethanol oxidation rate was measured as non-volatile products plus CO_2 formed from 1-^{14}C-ethanol (6).

Results

Rat liver cells oxidize ethanol at a rate comparable to that of isolated rat liver (7,8). Two (or more) pathways seem to be involved when high concentrations of ethanol are present (6,9-11). Both pathways will produce acetaldehyde (see Fig. 2).

The rate of ethanol oxidation by isolated rat liver cells as a function of ethanol concentration is shown in Figure 1. The rate of ethanol oxidation increases markedly as the ethanol concentration rises. Addition of 18 mM pyrazole to the incubation mixture gives rise to intracellular pyrazole concentrations of probably the same magnitude (11a) and thus an almost complete inhibition of ADH (about 96% at 60 mM ethanol). In spite of that, ethanol is still oxidized at a considerable rate at these high concentrations. This may be explained by the existence of an ethanol oxidizing system in the liver cells in addition to ADH (cf. Fig. 2).

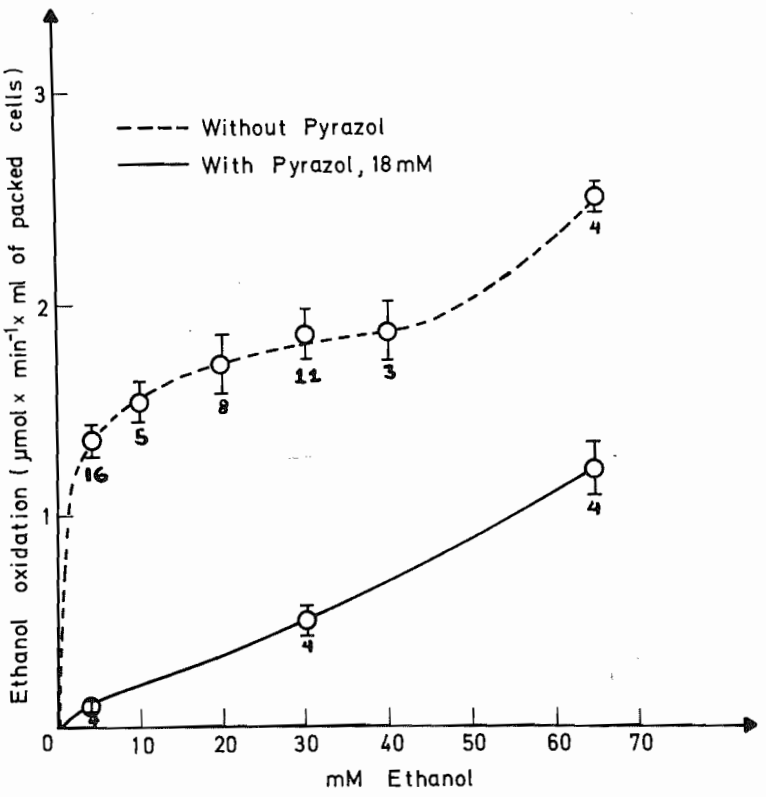

Figure 1. Ethanol oxidation rate by isolated rat liver parenchymal cells versus ethanol concentration. Cells were incubated in the buffer described in the "Methods" section. The oxidation rate represents the average rate over 20 min of incubation. Bars indicate S.E. and figures below the bars the number of cell preparations.

Up to a concentration of 40 mM the ethanol oxidation in rat liver cells may be explained by a combined action of two ethanol oxidizing systems: an ADH system with V_{max} 1.5 μmol x min^{-1} x wet wt. of packed cells and Km 0.5 mM ethanol and an altervative ethanol oxidizing system with V_{max} 0.6 μmol x min^{-1} x wet wt. and Km 10 mM (7).

The cytosolic $NADH/NAD^+$ ratio in isolated rat liver cells is greatly increased in the presence of 60 mM ethanol (Table I), as indicated by the high lactate/pyruvate ratio.

TABLE I

LABELING PATTERN OF LACTATE AND β-HYDROXYBUTYRATE IN ISOLATED RAT LIVER PARENCHYMAL CELLS

	Ethanol (60 mM) +Pyruvate (3 mM)	Ethanol (60 mM) +Pyruvate (3 mM) +Pyrazole (18mM)
Lactate, mM	1.40	0.65
Pyruvate, mM	0.089	1.01
Lactate/Pyruvate	49.0	0.71
β-Hydroxybutyrate, mM	0.17	0.051
Acetoacetate, mM	0.66	0.64
β-Hydroxybutyrate/ Acetoacetate	0.28	0.072
Specific activity of lactate relative to that of ethanol	0.12	0.08
Specific activity of β-hydroxybutyrate relative to that of ethanol	0.25	0.20

Cells incubated with [1-^{3}H]ethanol for 5 min. Figures represent means of three experiments, performed as described in the "Methods" section. The specific activity of ethanol was 160-170 cpm/nmol.

Addition of 18 mM pyrazole reverses the increase in the ratio. The total amount of lactate plus pyruvate, however, remains unchanged. The β-hydroxybytyrate/acetoacetate ratios are also decreased by the addition of pyrazole (Table I).

In the presence of 3 mM pyruvate there was incorporation of ^3H from [1-^3H]ethanol into lactate, the specific activity of lactate being 8-12% that of ethanol. The specific activity of lactate was not significantly lowered by addition of pyrazole. This may be due to extensive dilution of cytosolic NAD^3H by NADH from other cytosolic NADH-generating processes or to non-redox equilibrium between the ADH and the LDH reaction.

In experiments without added pyruvate the lactate concentration in the medium was very low, about 0.1 mM, and no incorporation of ^3H into lactate could be found.

The relative specific activity of β-hydroxybutyrate was about two times higher than the specific activity of lactate (Table 1).

Discussion

Generation of tritium-labeled NADH during metabolism of [1-^3H]ethanol by isolated liver cells. We have attempted to estimate the site of acetaldehyde oxidation by measuring the incorporation of ^3H from [1-^3H]ethanol into lactate and into β-hydroxybutyrate. The tritium-labeled ethanol is used as a substrate in order to generate a constant physiologically low concentration of [1-^3H]acetaldehyde during the experiments.

Both ADH and the alternative ethanol oxidizing systems will produce [1-^3H]acetaldehyde. However, [4A-^3H]NADH will only be produced in the ADH-catalyzed reaction (Fig. 2). If an isotope effect is absent, the specific activity of NAD^3H and of tritium-labeled acetaldehyde produced by ADH will be identical and half that of ethanol.

The oxidation of [1-^3H]ethanol by a non-ADH pathway will not produce tritium-labeled NADH, as half of the ^3H label will be incorporated into acetaldehyde and the other half into water (Fig. 2). The oxidation of [1-^3H]acetaldehyde will produce [4A-^3H]NADH of the same specific activity as acetaldehyde [Fig. 2 and (12)].

It follows from the consideration above that if [1-^3H] ethanol is metabolized exclusively by the non-ADH pathway (e.g., when the ADH-mediated ethanol oxidation is inhibited by sufficiently high pyrazole concentrations) the intracellular location of the formation of [4A-^3H]NADH will depend on whether acetaldehyde is oxidized intra- or extramitochondrially.

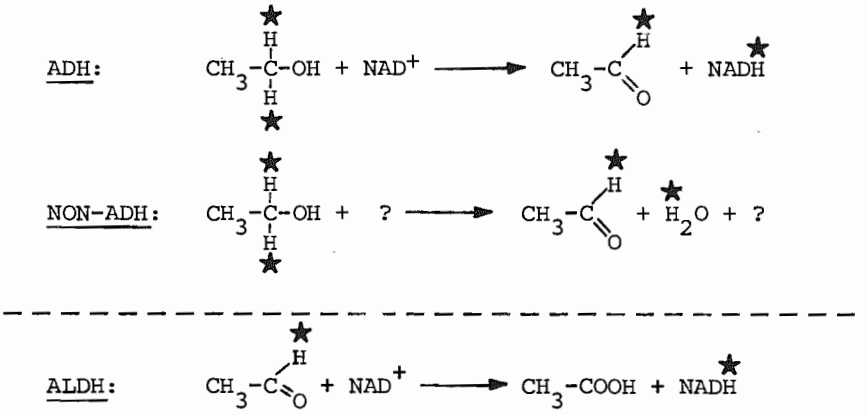

Figure 2. Pathways of ethanol to acetate in isolated rat liver parenchymal cells. ★ indicates a tritium atom. The ADH pathway produces labeled acetaldehyde and isolated NADH. In contrast, the NON-ADH pathway(s) does not produce labeled coenzymes, as half of the label goes to acetaldehyde, the other half to water. The oxidation of acetaldehyde formed by these reactions will produce labeled NADH. The intracellular location of the formation of labeled NADH will therefore be the intracellular location of acetaldehyde oxidation if the ADH pathway is totally inhibited.

Much evidence supports the assumption that in rat liver near-equilibrium conditions exist between the bound NAD^+/NADH, the free NAD^+/NADH and lactate/pyruvate in the cytosolic space or β-hydroxybutyrate/acetoacetate in the mitochondrial space. When [1-^{3}H]acetaldehyde is metabolized, the ^{3}H from the tritium-labeled NADH might be found in lactate or in β-hydroxybutyrate depending on where in the liver cell acetaldehyde is metabolized. The degree of labeling of lactate and of β-hydroxybutyrate will depend on the contribution of other substrates to NAD^+ reduction in cytosol and mitochondria, on the exchange of label between cytosol and mitochondria and on isotope effects in the overall transfer of ^{3}H from [1-^{3}H]ethanol to lactate and to β-hydroxybutyrate.

The above considerations are independent of the stereospecificity of the dehydrogenases involved as long as an

intact cell preparation is considered (12a). If only the dehydrogenases involved in the transfer of tritium from ethanol to lactate and β-hydroxybutyrate are present, however, these enzymes [ADH, ALDH (aldehyde dehydrogenase), LDH and HBDH (hydroxybutyrate dehydrogenase)] should all exhibit the same stereospecificity with respect to NADH. ADH, cytoplasmic ALDH and LDH have been reported to be A-specific in the transfer of hydrogen to NAD^+ (13). We have found the HBDH [both commercially obtained (Boehringer-Mannheim) and from rat liver mitochondria] to have the same specificity (the relative specific activity of β-hydroxybutyrate after incubation of [1-^{3}H]ethanol with NAD^+, ADH, acetoacetate and commercial HBDH or sonicated rat liver mitochondria plus rotenone was 0.35-0.43 and 0.20-0.28 respectively. Hoberman has reported the HBDH to be B-specific (13a).

The oxidation of racemically labeled [1-^{3}H]ethanol by perfused livers occurs without a large primary isotope effect, R*/R ratio (ratio between ^{3}H and ethanol fluxes) being about 0.6 as reported by Hobermann at this Symposium, the same value as obtained for the K_T/K_H ratios in experiments with the isolated enzyme (14). The isotope effect for the reaction NADH + pyruvate → NAD^+ + lactate is not known when the reaction proceeds in the liver, but for the isolated enzyme the K_T/K_H ratio in the presence of 3 mM pyruvate is about 0.9 (15). From this we may infer that if the oxidation of acetaldehyde is localized in the cytosol and if the metabolism of ethanol is mediated by a non-ADH system only, the specific activity of tritium-labeled lactate will be 0.5 times the specific activity of the [1-^{3}H]ethanol multiplied by the isotope effect (e.g., 0.6 x 0.9) on the overall transfer of tritium from ethanol to lactate or less [the specific activity will decrease if isotope dilution occurs, e.g., through a partial equilibration of the cytosolic and mitochondrial NADH (16)].

The specific activity of lactate and of β-hydroxybutyrate is about 10% and 20-25% respectively of the specific activity of the labeled ethanol, both in the absence and in the presence of pyrazole (Table I). These results, and especially the insensitivity of the labeling to inhibition of ADH, support the concept of an intramitochondrial oxidation of acetaldehyde. However, if acetaldehyde oxidation should occur exclusively in the mitochondria, the mitochondrial NAD^3H should be transferred to the cytosol without a substantial isotope dilution and without appreciable loss

of label in order to account for the labeling of the lactate pool. If only the ADH activity should account for the formation of cytoplasmic NAD^{3}H the specific activity of lactate should be almost zero when pyrazole is added. This is not the case (Table I) and we consider it probable that at least some labeling of lactate is due to cytoplasmic oxidation of acetaldehyde.

The specific activity of β-hydroxybutyrate is higher than that of lactate (Table I), which is not to be expected if the ^{3}H in β-hydroxybutyrate comes from ctyosolic NAD^{3}H (16,17). The present results, therefore, indicate that acetaldehyde oxidation occurs both in the cytosolic and the mitochondrial compartments of the liver cell.

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